

## REMARKS

The Applicant thanks the Examiner for examining claims 1 – 17, 36, and 48 – 56, and respectfully requests reconsideration of these claims in light of the amendments and arguments submitted herewith.

### *Claim Objections*

Claims 17 and 56 are amended to attend to grammatical irregularities pointed out by the Examiner; the amendments do not introduce any narrowing of the scope of claims 17 and 56.

### *Indefiniteness*

Claims 1 – 11 and 48 were rejected under 35 U.S.C. §112, second paragraph for being indefinite. Claim 1 -10 are amended to address the Examiner's concerns. In particular, the changing of the preamble of claims 2 -10 presents no narrowing of the scope of the claims.

Claim 11 is amended to include the limitation that the “DNA sequence is operably linked to a promoter” as requested by the Examiner. The claim, however, is not amended with regard to specific targeting of an enhancer or promoter to a specific gene expression. Claim 11 requires “introducing . . . a DNA sequence operably linked to a promoter and corresponding to at least one of an enhancer and a gene so as to alter gene expression.” An “expression altering sequence” as defined in the application (lines 1 – 19, page 10) has activity that may be broader than targeting “specific gene expression”. Even to the extent that a promoter or enhancer is specifically targeted, such is understood by those skilled in the art. Thus, the claim requires no further amendment.

The office action rejects claim 48 under §112, stating that the term *derivative* is unclear. The claim is amended to clarify that derivative cells are “cells derived from transfected human embryonic stem cells”.

The office action also rejects claim 48, stating that the term *foreign* is unclear. The term, however, is clear in the context of the claim. Claim 48 includes the element of “pluripotent human embryonic stem cells modified by *foreign genetic material which is . . .*” That is, the claim explains what is included in the term *foreign genetic material*. The

word *exogenous* is inappropriate since its meaning may contradict language in claim 48 that DNA “which occurs in the embryonic stem cells but is not expressed in them at levels which are biologically significant” may be *foreign genetic material*.

Considering the amendments and arguments presented, the Applicant submits that pending claims 1 – 17, 36, 48 – 56 are clear, complete, and definite in accord with the requirements of 35 U.S.C. §112, second paragraph.

### *Prior Art Rejections*

The Applicant submits that the pending claims are drawn to novel features that are not taught by the cited art, and as such are allowable over the prior art rejections stated in the office action.

A. The Unexpected Success of Using Cationic Polymer Reagents to Transfect Human Embryonic Stem Cells is Not Suggested or Taught by the Cited Art

As discussed from line 24, page 11 to line 18, page 12 of the detailed description, and depicted in Figure 1, the application reveals an increase in relative activity for a transfected gene in the neighborhood of an order of magnitude using cationic polymer reagents relative to other transfection techniques as applied to human embryonic stem (hES) cells. This result enables the introduction of polynucleotides into hES cells, and the production of cell populations comprising transfected hES cells, relative to other techniques that are shown to be ineffective. The result is also unexpected; the efficiency of various transfection techniques differs according to the type of embryonic stem (ES) cell transfected. As stated in the application at lines 28 – 33 of page 11, electroporation was found to be the method of choice for murine ES cells. In contrast for hES cells, cationic polymer reagents provide dramatically better efficiencies as measured by relative activity of the transfected polynucleotide. None of the cited art suggests or teaches this unexpected result identified by the application.

1. Claims 7 and 11 - 17

Given the unexpected result taught by the application, claims 7 and 11 – 17 are clearly patentable over the prior art rejections. Claim 7 is drawn to a method of altering gene expression requiring “a cationic non-lipid polymer transfection reagent for

introducing the polynucleotide into the population of cells.” Claim 11 is amended to strike the limitation related to electroporation. Thus the method of amended claim 11 requires the step of introducing a DNA sequence into a population of human embryonic stem (hES) cells “in the presence of a cationic polymer . . .” Claims 12 – 17 depend from claim 11.

Claims 11 – 17 are currently rejected under 35 U.S.C. § 102(b) with respect to Smith et al. (U.S. Patent No. 6,146,888). Smith et al., however, provides no teaching of the “presence of a cationic polymer” as required by amended claims 11 – 17. As such, Smith et al. cannot anticipate these claims.

Claims 7 and 11 – 17 are also currently rejected under 35 U.S.C. § 103(a) in light of various combinations of Smith et al.; Myers (Molecular Biology and Biotechnology, ed. Myers, VCH Publishers, Inc., 1995, pp. 165 - 168); Fasbender et al. (Jour. Biol. Chem., 272(10):6479 – 6489, 1997); Pascolo et al. (J. Exp. Med., 185:2043 – 2051, 1997); Thomson (Science, 282:1145 – 1147, 1998); and Bradley et al. (U.S. Patent No. 5,614,396). None of the cited art, with the exception of Fasbender et al., teaches the use of cationic polymer reagents for transfection of hES cells. Thus combinations of these references do not teach the necessary element of cationic polymer reagents required in claims 7 and 11 – 17. As such, the references, alone or in combination, cannot support a prima facie case of obviousness.

A combination of Fasbender et al. with Smith et al. also cannot support a obviousness rejection. Fasbender et al. is drawn to using a cationic polymer, or a cationic lipid, with an adenovirus to infect human epithelia or nasal epithelium of cystic fibrosis in mice (see abstract). Fasbender et al. provides no teaching of the unexpected superiority of cationic polymer reagents in transfecting human ES cells.

Fasbender et al. actually teaches away from the unexpected benefits of cationic polymer reagents in two ways. First, the reference shows an improvement in gene transfer using cationic *lipids or polymers*; Figure 4 in Fasbender shows that Lipofectamine works extremely well in transfecting fliosarcoma cells. In contrast, Figure 1 of the application shows that Lipofactamine works extremely poorly in transfecting hES cells, providing little if any improvement relative to a control measurement. Thus

Fasbender et al. teaches away from the superiority of cationic polymers relative to cationic lipids.

Second, Fasbender et al. does not distinguish between mouse and human cells, and does not show its techniques work on ES cells. Fasbender et al. shows results in relation to *human epithelial* cells and *murine nasal epithelium* cells. Thus, the reference does not indicate the success of cationic polymer reagents in transfection of *human embryonic stem* cells.

Moreover, Fasbender et al. points away from a reasonable expectation of success in claims 7 and 11 – 17 since the reference requires the presence of an adenovirus vector with the cationic vector to catalyze gene transfer. The Fasbender et al.'s abstract states that “[n]onviral cationic vectors bind efficiently to the negatively charged cell surface, *but do not catalyze subsequent steps in gene transfer*” (emphasis added). Claims 7 and 11 – 17, however, do not require the presence of an adenovirus. In fact, Figure 1 of the application presents results showing activity due to transfection techniques that do not utilize an adenovirus.

Thus the cited art fails to anticipate or render obvious claims 7 and 11 – 17. The claims are allowable.

## 2. Claims 36 and 48 - 56

Moreover, claims 36 and 48 – 56 are not anticipated or obvious in light of the prior art because the cited art fails to enable the creation of the claimed subject matter. Claim 36 is drawn to “a substantially pure population of human embryonic stem (hES) cells containing an expression altering sequence of exogenous DNA.” Claims 48 – 56 are drawn to “[a] reagent cell population . . . of pluripotent human embryonic stem cells modified by foreign genetic material.” The cited art, however, does not enable such claims because transfection rates taught by the transfection techniques of the cited art are too low to create the claimed cell populations.

The evidence provided in the examples of Smith et al. (see Examples in cols. 6 – 12) only document the use of electroporation protocols to insert DNA into murine embryonic stem (ES) cells. Zwaka and Thomson showed that murine electroporation protocols, to insert DNA into hES cells, are ineffective (see “Homologous recombination in human embryonic stem cells,” *Nature Biotechnology*, Vol. 21, pp. 319 – 321, March

2003 (a copy of which is included for the Examiner's review)). They concluded that "[g]iven current culture techniques, [the transfection rate] is too low to be practical for identifying rare homologous recombination events" (see column 2, first full paragraph, page 319). Smith et al. states without showing that injection, lipofection, transfection, and infection with a viral vector may be used. The reference, however, provides no showing that these techniques enable transfection into human ES cells at rates substantial enough to create the claimed cell populations. Thus, Smith et al. cannot anticipate claims drawn to a "substantially pure" or "reagent" cell population of hES cells with foreign genetic material because the reference does not enable the making of such populations; identification of the altered hES cells using Smith et al. is not shown.

Combinations of Smith et al. with the other cited art also cannot provide an obviousness rejection for claims 36 and 48-56. None of the references, with the exception of Fasbender et al., teaches a transfection technique capable of providing the claimed subject matter, i.e. hES cell populations with the exogenous DNA or foreign genetic material. To the extent that Fasbender et al. teaches such a technique, it cannot be combined with Smith et al. for the reasons stated earlier.

Thus claims 36 and 48-56 are also allowable over the prior art rejections.

B. Protocols for Transfecting Human Embryonic Stem Cells are Neither Taught Nor Suggested by the Cited Art

As stated in the application, "ES cell lines other than those of mice have not be transfected with exogenous DNA" (see lines 24 – 28 on page 11). Given the differences between murine and human embryonic stem cells, the cited art neither anticipates nor renders obvious any of the pending claims of the application.

1. Homologous Recombination Techniques Fail with Human Embryonic Stem Cells as of the Applicant's Priority Date

The office action rejected claims 1 – 4, 6, 10 – 12, 15, 16, 36, 48, 52, 54, and 56 under 35 U.S.C. § 103(a), asserting that a combination of Thomson and Bradley et al. renders the claims obvious. In summary, Thomson teaches the harvesting of human ES

cells from the inner cell masses of blastocysts and Bradley et al. teach the transfection of embryonic stem cells using electroporation and homologous recombination. Though Bradley et al. state that the invention may be performed with human cells, the data presented only show homologous recombination using the AB1 cell line, a murine embryonic cell line (see Examples in cols. 29 – 36).

No reasonable expectation of success exists to combine these references because others have shown that homologous recombination techniques, known at the time of filing and applied to human ES cells, fail to produce transfected human ES cells. In particular, Zwaka and Thomson have shown that using a typical mouse ES cell protocol to transfect human ES cells to induce homologous recombination yielded a stable transfection rate of  $10^{-7}$  (see page 319, right-hand column, first full paragraph); a rate which is “too low to be practical for identifying rare homologous recombination events.” Though Zwaka and Thomson delineate inventive concepts for electroporating human ES cells for homologous recombination, the concepts are published in February 2003, several years following the current application’s priority date. Thus as of the priority date of the application, combining Thomson and Bradley et al. would not have been sufficient to render any of the pending claims obvious.

2. None of the Cited Art Shows A Successful Protocol for Transfecting Human Embryonic Stem Cells

Claims 1 – 17, 36, and 48 – 56 are all drawn to methods of altering gene expression in human ES cells, and compositions including human ES cells that contain an expression altering sequence or foreign genetic material. The cited prior art teaches the extraction of human ES cells (Thomson) or techniques for introducing DNA into cells other than human ES cells. However, the cited art fails to support a rejection of anticipation or obviousness because none of the cited art is drawn toward methods or compositions involving *human* embryonic stem cells that contain an expression altering sequence or foreign genetic material.

The cited art provides no protocols for DNA transfection into embryonic stem cells of any specie other than mice. Smith et al. may state that some of its techniques are applicable to humans, but all the data provided by the examples are limited to mouse cells. Myers, Fasbender et al., and Pascolo et al. provide elements of genes that may be

transferred or transfection techniques, but they provide no insight as to the success of their teachings as applied to human embryonic stem cells.

The lack of any previous protocol for DNA transfection into human embryonic stem cells is significant because it underscores that murine ES cell techniques cannot be assumed to work with human ES cells. As discussed earlier, electroporation may be a successful transfection technique for murine ES cells, but the technique has extremely poor efficiency when applied to human ES cells. Also, homologous recombination techniques as taught by Bradley et al. may have some success with murine ES cells, but as Zwaka and Thomson have shown, such homologous recombination techniques as applied to human ES cells are unsuccessful.

As well, substantial other differences exist between mouse and human embryonic stem cells. As discussed by Kaufman et al., “mouse and human ES cells differ in morphology, population doubling time, and growth factor requirements” (“Hematopoietic Colony-Forming Cells Derived from Human Embryonic Stem Cells,” PNAS Online, Vol. 98, No. 19, pp. 10716 – 10721, September 2001 (a copy of which is included)). For example, mouse ES cells can be maintained in an undifferentiated state in the absence of feeder cells if the cell media includes leukemia inhibitory factor (LIF). In contrast, hES cells will either differentiate or die if exposed to LIF without feeder cells (id.).

All these differences show that one skilled in the art cannot expect that techniques that succeed in producing a particular result with murine ES cells will produce a similar result in human ES cells. Indeed, the specific failures of electroporation and homologous recombination in human ES cells point away from a reasonable expectation of using murine transfection techniques in human ES cells.

Thus Smith et al. cannot anticipate any of the pending claims. The reference only enables its techniques for murine ES cells, not human ES cells as required by every pending claim. As well, Smith et al. in combination with any of Myers, Fasbender et al., and Pascolo et al. cannot render any of the pending claims obvious because one skilled in the art could not have a reasonable expectation of success given the substantial differences between human and murine ES cells; none of the references even attempt to specifically address the differences between human and murine ES cells.

Thus claims 1 – 17, 36, and 48 – 56 are patentable over the prior art rejections presented in the office action.

*Conclusion*

In view of the amendments and arguments presented, the Applicant respectfully requests allowance of pending claims 1 – 17, 36, and 48 – 56. The representative of the Applicant cordially requests a telephone conference with the Examiner to discuss this paper, if such communication would help expedite the prosecution of the application.

Respectfully submitted,

A handwritten signature in black ink, appearing to read 'Charlton Shen', with a stylized flourish at the end.

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# Homologous recombination in human embryonic stem cells

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Homologous recombination applied to mouse embryonic stem (ES) cells has revolutionized the study of gene function in mammals<sup>1-4</sup>. Although most often used to generate knockout mice, homologous recombination has also been applied in mouse ES cells allowed to differentiate *in vitro*. Homologous recombination is an essential technique if human ES cells<sup>5</sup> are to fulfill their promise as a basic research tool. It also has important implications for ES cell-based transplantation and gene therapies. Significant differences between mouse and human ES cells have hampered the development of homologous recombination in human ES cells. High, stable transfection efficiencies in human ES cells have been difficult to achieve, and, in particular, electroporation protocols established for mouse ES cells work poorly in human ES cells<sup>6</sup>. Also, in contrast to their murine counterparts, human ES cells cannot be cloned efficiently from single cells, making it difficult to screen for rare recombination events<sup>7</sup>. Here we report an electroporation approach, based on the physical characteristics of human ES cells, that we used to successfully target *HPRT1*, the gene encoding hypoxanthine phosphoribosyltransferase-1 (*HPRT1*), and *POU5F1*, the gene encoding octamer-binding transcription factor 4 (Oct4; also known as POU domain, class 5, transcription factor 1 (*POU5F1*)).

The *HPRT1* gene is located on the X chromosome, so a single homologous recombination event leads to complete loss of function in XY cells. *HPRT1*-deficient cells can be selected based on their resistance to 2-amino-6-captapurine (6-TG), and thus the frequency of homologous recombination events is easy to estimate<sup>8</sup>. Because of these properties, *HPRT1* played an important role in the initial development of homologous recombination in mouse ES cells<sup>4,9</sup>. We designed an *HPRT1*-targeting vector that contains a short homologous arm (1.9 kb) on the 5' side of exon 7 and a long homologous arm (10 kb) on the 3' side of exon 9, which deletes regions of the last three exons (Fig. 1A). A neomycin resistance (*neo*) cassette was inserted between the two homologous arms, and at the end of the 3' homologous arm, the thymidine kinase gene (*tk*) was added to allow negative selection with gancyclovir.

For human ES cells, the best chemical reagents yield stable (drug-selectable) transfectants at rates of about 10<sup>-5</sup>; mouse ES cell electroporation procedures yield substantially lower rates<sup>6</sup>. Given the very low transfection rates previously reported for electroporation, we first tested two chemical transfection reagents (ExGen 500 versus FuGene-6) for homologous recombination of the *HPRT1* locus in human ES cells. Although clones were obtained using both transfection reagents—for ExGen and FuGene respectively, 130 versus 261 G418-resistant clones and 35 versus 61 gancyclovir-resistant clones—none of these were resistant to both G418 and 6-TG (*HPRT1*<sup>-</sup>), indicating that none were the result of homologous recombination. These

results are consistent with the observation that transfection using lipid (FuGene-6; Roche, Indianapolis, IN) and cationic (ExGen 500; Fermentas, Hanover, MD) reagents results in inefficient homologous recombination in other mammalian cell types, and that physical methods of introducing DNA are, in general, more effective<sup>10</sup>.

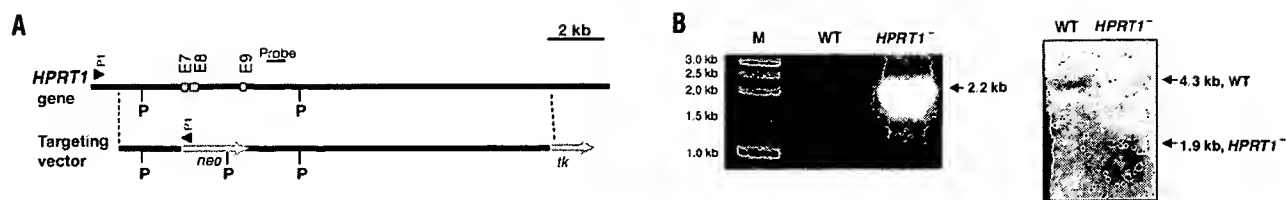
Our failure to achieve homologous recombination with chemical transfection reagents led us to re-evaluate electroporation procedures for human ES cells. In our hands, electroporation using a typical mouse ES cell protocol<sup>11</sup> (220 V, 960  $\mu$ F, electroporation in PBS) yielded a stable transfection rate of  $\sim 10^{-7}$ . Given current culture techniques, this frequency is too low to be practical for identifying rare homologous recombination events. As human ES cells are significantly larger than mouse ES cells ( $\sim 14 \mu$ m versus  $\sim 8 \mu$ m), we tried electroporation parameters described for larger cells. Also, because our current culture conditions allow only about 1% of individual human ES cells to survive and form colonies when plated at low densities, we electroporated the ES cells in clumps, not as individual cells, and plated them out at high densities. Additionally, we electroporated the cells in an isotonic, protein-rich solution (standard cell culture medium), instead of PBS, at room temperature. Using this modified protocol, we were able to obtain stable, G418-resistant clones at transfection rates that were 100-fold (or more) higher than those attained with standard mouse ES cell electroporation procedures. After transfection of  $1.5 \times 10^7$  cells with the linearized *HPRT1*-targeting vector, we obtained 350 G418-resistant clones. Of these, 50 were resistant to gancyclovir, and of these, 7 were also resistant to 6-TG, suggesting successful homologous recombination. Polymerase chain reaction (PCR) and Southern blotting (Fig. 1B) confirmed that homologous recombination had occurred in all of these 6-TG-resistant clones.

One of the uses of homologous recombination in human ES cells will be to generate 'knock-in' cell lines with a selectable marker introduced into a locus with a tissue-specific expression pattern. Such knock-ins will be useful, for example, to purify a specific ES cell-derived cell type from a mixed population<sup>12,13</sup>. To test this approach, we introduced two reporter genes into the Oct4-encoding gene *POU5F1* by homologous recombination. Oct4, which belongs to the POU (Pit, Oct, Unc) family of transcription factors<sup>14</sup>, is expressed exclusively in the pluripotent cells of the embryo and is a central regulator of pluripotency<sup>14,15</sup>. We introduced two promoterless reporter-selection cassettes into the 3' untranslated region (UTR) of *POU5F1*. The first cassette contained an internal ribosomal entry site (IRES) sequence of the encephalomyocarditis virus and the gene *EGFP*, encoding the enhanced green fluorescence protein (EGFP). The second cassette included the same IRES sequence and the gene *neo*, encoding neomycin resistance. The cassettes were flanked by two homologous arms (Fig. 2A). After electroporation of  $1.5 \times 10^7$  human ES cells with the linearized targeting vector (Fig. 2A), we obtained 103 G418-resistant clones. PCR (Fig. 2B, left) and DNA Southern blotting (Fig. 2B, right) demonstrated that 28 of these clones (27%) were positive for homologous recombination. Using a second targeting vector with a longer 3' homologous arm, we obtained a higher rate of homologous recombination, almost 40% (22 homologous clones out of 56 G418-resistant clones). Similar transfection experiments using FuGene-6 with the same *POU5F1*-targeting vector resulted in 11 G418-resistant clones, none of which resulted from homologous recombination.

Human ES cells with the *POU5F1* knock-in expressed EGFP (Fig. 2C), which turned off during differentiation. Both drug selection and flow cytometry to detect EGFP expression (Fig. 2D) allowed purification of undifferentiated ES cells from a mixed, partially differentiated cell population. These properties will make the knock-in cell lines useful for studying *POU5F1* gene expression during differentiation *in vitro* and for optimizing culture conditions for human ES cells.

Electroporation of human ES cells with a DNA construct containing a *neo* cassette under the control of the *tk* promoter yielded a stable

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**Figure 1.** Targeted deletion of the last three exons of the *HPRT1* gene. (A) Partial structure of human *HPRT1* and the gene-targeting vector. 3' probe for Southern blot analysis is shown. E, exon; P, *Pst*I; P1, primer pair; *tk*, thymidine kinase gene. (B) PCR analysis of ES cell lines with P1 (left) and Southern blot analysis with dedicated probe and *Pst*I digest (right). *HPRT1*<sup>-/-</sup>, knockout; WT, wild-type cells; M, marker.

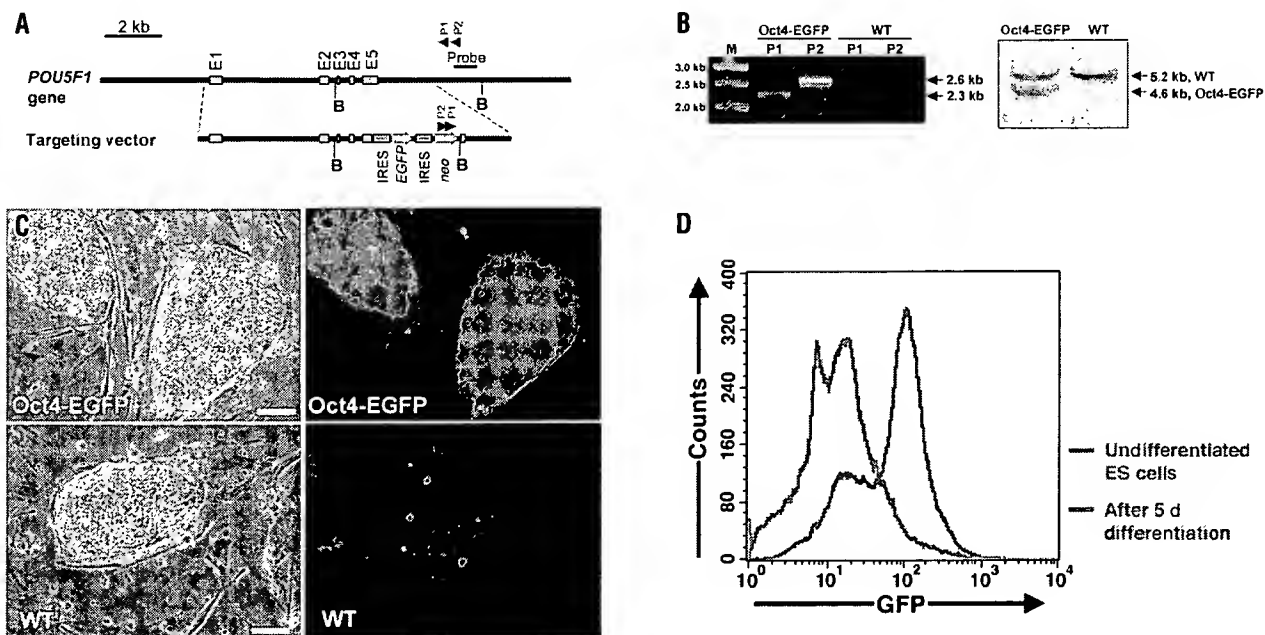
transfection rate of  $5.6 \times 10^{-5}$ , giving an estimated 26:1 ratio of stable transfected clones to homologous recombination events for the first *POU5F1* construct. Similarly, for transfection of the *HPRT1* vector, the ratio of G418-resistant clones to *HPRT1*<sup>-/-</sup> clones was 50:1. These targeting ratios for both *HPRT1* and Oct4 are comparable to those observed for mouse ES cells<sup>16</sup>, and suggest that although successful transfection strategies differ between human and mouse ES cells, the frequency of homologous recombination itself may be similar. However, it will be important to determine whether this similarity of rates between human and mouse ES cells holds true for genes not expressed in ES cells.

Homologous recombination in human ES cells will be important both for elucidating gene function *in vitro* and for modifying specific ES cell-derived tissues for therapeutic applications in transplantation medicine. For therapeutic applications, controlled modification of specific genes should be useful for purifying specific ES cell-derived differentiated cell types from a mixed population, for altering the antigenicity of cells, and for giving cells new properties (such as viral resistance) to combat specific diseases. Homologous recombination in human ES cells might also be used for recently described approaches combining therapeutic cloning with gene therapy<sup>17</sup>. Modifying specific genes for *in vitro* studies will be important for learning more about

the pathogenesis of diseases for which mouse models have proven inadequate. For example, *Hprt1*-deficient mice do not show a phenotype similar to Lesch-Nyhan syndrome, the condition that results from *HPRT1* deficiency in humans<sup>18</sup>. *In vitro* neural differentiation of *HPRT1*<sup>-/-</sup> human ES cells or transplantation of ES cell-derived neural tissue to an animal model<sup>19</sup> could help clarify the pathogenesis of Lesch-Nyhan syndrome. Indeed, homologous recombination and human ES cells offer a promising approach for understanding the function of any human gene, and this approach will be particularly important for human genes that differ in clinically significant ways from the corresponding mouse genes.

### Experimental protocol

***HPRT1* knockout.** The gene-targeting vector was constructed by replacement of the last three exons (exon 7, 8, and 9) of the *HPRT1* gene with a *neo* cassette under the control of the *tk* promoter. This cassette is flanked in the 5' direction by a 10 kb homologous arm and in the 3' direction by a 1.9 kb homologous arm. Isogenic homologous DNA was obtained by long-distance genomic PCR and subcloned. H1.1 human ES cells were cultured as described<sup>5</sup>. One week before electroporation, cells were plated onto Matrigel (Becton Dickinson, San Jose, CA) and cultured with fibroblast-conditioned medium<sup>20</sup>. To remove colonies as intact clumps, human ES cell cultures were treated with collagenase



**Figure 2.** Targeting of an IRES-EGFP-IRES-neo cassette into the 3' UTR of the gene *POU5F1*, which encodes Oct4. (A) Partial structure of the human *POU5F1* gene and the gene-targeting vector. 3' probe for Southern blot analysis is shown. E, exon; B, *Bam*HI; P1 and P2, primer pairs 1 and 2. (B) PCR analysis of ES cell lines with P1 and P2 (left) and Southern blot analysis with dedicated probe and *Bam*HI digest (right). *POU5F1*-EGFP, heterozygous knock-in; WT, wild-type cells; M, marker. (C) Fluorescence microscopy (right) and phase-contrast microscopy (left) of *POU5F1* knock-in and wild-type colonies. Bar, 25  $\mu$ m. (D) Flow cytometry of *POU5F1* knock-in undifferentiated (EGFP-positive) ES cells (blue) and their differentiated derivatives after 5 d of differentiation (red).

IV (1 mg/ml; Invitrogen, Carlsbad, CA) for 7 min, washed with medium, and resuspended in 0.5 ml culture medium ( $1.5\text{--}3.0 \times 10^7$  cells). Just before electroporation, 0.3 ml PBS (Invitrogen) containing 40  $\mu$ g linearized targeting vector DNA was added. Cells were then exposed to a single 320 V, 200  $\mu$ F pulse at room temperature using the BioRad Gene Pulser II (0.4 cm gap cuvette; BioRad, Hercules, CA). Cells were incubated for 10 min at room temperature and were plated at high density on one 10 cm culture dish coated with Matrigel. G418 selection (50  $\mu$ g/ml, Invitrogen) was started 48 h after electroporation. After one week, G418 concentration was doubled and 6-TG selection (1 mM; Sigma, St. Louis, MO) was started. After three weeks, surviving colonies were analyzed individually by PCR using primers specific for the *neo* cassette and for the *HRPT1* gene just upstream of the 5' homologous region, respectively. PCR-positive clones were rescreened by Southern blot analysis using *Pst*I-digested DNA and a probe on the 3' side of the *neo* cassette.

**POU5F1 knock-in.** The gene-targeting vector was constructed by insertion of an IRES-EGFP, an IRES-*neo*, and an SV40 polyadenylation sequence (approximately 3.2 kb) into the 3' untranslated region of the fifth exon of the human *POU5F1* gene. This cassette is flanked in the 5' direction by a 6.3 kb homologous arm and in the 3' direction by a 1.6 kb (6.5 kb in an alternative targeting vector) homologous arm. Isogenic homologous DNA was obtained by long-distance genomic PCR and subcloned. H1.1 human ES cells were cultured as described<sup>5</sup>. When an alternative targeting vector with a longer (6.5 kb) 3' homologous arm was used, the rate of homologous recombination increased to almost 40% (22 homologous clones out of 56 stable clones).

**Flow cytometry.** Before flow cytometry, ES cell differentiation was induced by incubating the cells for 5 d in unconditioned medium on Matrigel. ES cells were treated with trypsin-EDTA and washed with PBS (both from Invitrogen). Dead cells were excluded from analysis by forward- and side-scatter gating. Samples were analyzed using a FACScan (Becton Dickinson) flow cytometer and Cellquest software (Becton Dickinson). A minimum of 50,000 events was acquired for each sample.

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#### Competing interests statement

The authors declare that they have no competing financial interests.

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## Site-specific cassette exchange and germline transmission with mouse ES cells expressing $\phi$ C31 integrase

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Currently two site-specific recombinases are available for engineering the mouse genome: Cre from P1 phage<sup>1,2</sup> and Flp from yeast<sup>3,4</sup>. Both enzymes catalyze recombination between two 34-base pair recognition sites, *lox* and *FRT*, respectively, resulting in excision, inversion, or translocation of DNA sequences depending upon the location and the orientation of the recognition sites<sup>5,6</sup>. Furthermore, strategies have been designed to achieve site-specific insertion or cassette exchange<sup>7–10</sup>. The problem with both recombinase systems is that when they insert a circular DNA into the genome (*trans* event), two *cis*-positioned recognition sites are created, which are immediate substrates for excision. To stabilize the *trans* event, functional mutant recognition sites had to be identified<sup>8–12</sup>. None of the systems, however, allowed efficient selection-free identification of insertion or cassette exchange. Recently, an integrase from *Streptomyces* phage  $\phi$ C31 has been shown to function in *Schizosaccharomyces pombe*<sup>13</sup> and mammalian<sup>14,15</sup> cells. This enzyme recombines between two heterotypic sites: *attB* and *attP*. The product sites of the recombination event (*attL* and *attR*) are not substrates for the integrase<sup>16</sup>. Therefore, the  $\phi$ C31 integrase is ideal to facilitate site-specific insertions into the mammalian genome.

Here we demonstrate that the  $\phi$ C31 integrase system is compatible with embryonic stem (ES) cell-mediated genomic alterations in the mouse and is particularly useful to achieve site-specific transgene insertions or

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Cell Biology

## Hematopoietic colony-forming cells derived from human embryonic stem cells

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### ► Abstract

Human embryonic stem (ES) cells are undifferentiated, pluripotent cells that can be maintained indefinitely in culture. Here we demonstrate that human ES cells differentiate to hematopoietic precursor cells when cocultured with the murine bone marrow cell line S17 or the yolk sac endothelial cell line C166. This hematopoietic differentiation requires fetal bovine serum, but no other exogenous cytokines. ES cell-derived hematopoietic precursor cells express the cell surface antigen CD34 and the hematopoietic transcription factors TAL-1, LMO-2, and GATA-2. When cultured on semisolid media with hematopoietic growth factors, these hematopoietic precursor cells form characteristic myeloid, erythroid, and megakaryocyte colonies. Selection for CD34<sup>+</sup> cells derived from human ES cells enriches for hematopoietic colony-forming cells, similar to CD34 selection of primary hematopoietic tissue (bone marrow, umbilical cord blood). More terminally differentiated hematopoietic cells derived from human ES cells under these conditions also express normal surface antigens: glycophorin A on erythroid cells, CD15 on myeloid cells, and CD41 on megakaryocytes. The *in vitro* differentiation of human ES cells provides an opportunity to better understand human hematopoiesis and could lead to a novel source of cells for transfusion and transplantation therapies.

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## ▷ Introduction

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Embryonic stem (ES) cells are pluripotent cells derived from preimplantation embryos. ES cells have the ability to be maintained in culture indefinitely as undifferentiated cells, yet they are capable of forming more differentiated cell types. Because of these properties, mouse ES cells have been instrumental in gaining a better understanding of mammalian development. In studies of hematopoiesis, investigators have used mouse ES cells to derive various hematopoietic lineages *in vitro* either by formation of "embryoid bodies" (1, 2), coculture with stromal cell lines (3, 4), or culture on collagen-coated plates (5). These studies have used gene expression, cell phenotype, and functional studies to define sequential stages of hematopoietic cell development.

In contrast to work on mouse hematopoietic development, studies of human hematopoiesis have been confined to the use of primary hematopoietic tissue such as bone marrow, peripheral blood, or umbilical cord blood as the starting cell population. The reliance on these heterogeneous tissue samples that are difficult to sustainably expand *in vitro* has hindered progress in understanding human hematopoiesis. Work on human hematopoiesis typically uses cell surface antigens (such as CD34) to identify putative hematopoietic stem cell (HSC) population(s) within the mixed cell population, and cell sorting methods are used to enrich for the cells of interest (6, 7). Recently, fluorescent dyes that can bind DNA (such as Hoechst 33342) have proven useful in the differential isolation of putative HSCs (8). Although these methods have provided a great deal of information about HSC biology and have facilitated clinical hematopoietic cell transplantation, several important questions remain. For example, recent studies have shown that some CD34<sup>+</sup> cells also can display HSC properties, including long-term growth, differentiation, and self-renewal when injected into immunodeficient mice (8-11). Moreover, some cells derived from nonhematopoietic tissue appear to have HSC potential (12, 13). The interrelationship between these varying sources and phenotypes of HSCs remains unclear.

Human ES cells (14, 15) provide a unique, homogeneous, unlimited starting population of cells for studying human hematopoiesis. Human ES cells can be cultured for at least 300 population doubling times without observed senescence, while continuing to maintain normal karyotypes, telomere lengths, and pluripotency. Moreover, these cells can be cloned from a single cell without loss of pluripotency (16). Human ES cells give rise to differentiated cells and tissues from all three embryonic germ layers when allowed to form teratomas in immunodeficient mice or when induced to form embryoid bodies *in vitro* (14, 17). Mouse and human ES cells differ in morphology, population doubling time, and growth factor requirements. Undifferentiated mouse ES cells, for example, can be maintained as undifferentiated "feeder-independent" cells if growth factors such as leukemia inhibitory factor (LIF) or related cytokines are added to the media (1). If human ES cells are grown without feeder cells, but in the presence of LIF, they either differentiate or die (14, 15). Given the unexpected differences in the control of the undifferentiated proliferation of mouse and human ES cells, similar significant differences could exist in the specific factors that direct their differentiation.

Here, we show that coculture of human ES cells with certain stromal cell lines derived from mouse

hematopoietic tissue (yolk sac and bone marrow) leads to differentiation into hematopoietic cells. These cells express both cell surface antigens and transcription factors characteristic of cells in primary human hematopoietic tissue. Moreover, hematopoietic cells are present in these differentiating cocultures that can generate myeloid, erythroid, and megakaryocyte colonies *in vitro*, and the colonies obtained appear identical to those produced from human adult bone marrow cells.

## ► Methods

**Culture of ES Cells.** The human ES cell lines H1, H1.1, and H9.2 were derived and maintained as described (14, 16), except that the undifferentiated ES cells were grown in serum-free conditions. Human ES cells were maintained as undifferentiated cells by coculture with irradiated (25 Gy) mouse embryonic fibroblast (MEF) cells in media consisting of DMEM/F12 (GIBCO/BRL) supplemented with 15% KnockOut SR serum replacer (GIBCO/BRL), 2 mM L-glutamine, 0.1 mM  $\beta$ -mercaptoethanol, 1% nonessential amino acids (all from GIBCO/BRL), and 4 ng/ml basic fibroblast growth factor (R & D Systems). ES cells grown under these conditions will begin to show evidence of differentiation after  $\approx 10$  days; therefore, ES cells were passaged approximately weekly to maintain undifferentiated growth. To promote hematopoietic differentiation, the human ES cells were cocultured with either the mouse bone marrow stromal cell line S17 (18) (gift of Kenneth Dorshkind, University of California, Los Angeles) or the mouse yolk-sac endothelial cell line C166 (19). S17 and C166 cells were irradiated (30 Gy). Media to support differentiation consisted of DMEM (GIBCO/BRL) supplemented with 20% FBS (from either HyClone or Gencyte, Buffalo, NY), 2 mM L-glutamine, 0.1 mM  $\beta$ -mercaptoethanol, and 1% nonessential amino acids. During differentiation, media were changed every 2-3 days.

**Flow Cytometry Analysis.** Undifferentiated H1 cells were washed with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -free PBS and dissociated with 0.05% trypsin/0.53 mM EDTA (GIBCO/BRL) for 5-10 min before washing and staining with FACS media consisting of PBS supplemented with 2% FBS and 0.1% sodium azide. To analyze cell surface antigen expression on H1 cells allowed to differentiate as above (H1/S17 and H1/C166 cells), the differentiated cell mixture was dissociated with 1 mg/ml collagenase IV (GIBCO/BRL) and 0.05% trypsin/0.53 mM EDTA supplemented with 2% chick serum (GIBCO/BRL). Dissociated cells were filtered through 85- $\mu\text{m}$  nitex mesh to remove remaining clumps. The single cell suspension was aliquoted and stained with either isotype control or antigen-specific antibodies. Unconjugated isotype control antibodies IgG3 and IgM (Sigma) and directly conjugated isotype control antibodies IgG1-FITC and IgG1-phycoerythrin (PE) (PharMingen) were used. Unconjugated antigen-specific antibodies against SSEA-1 (IgM) and SSEA-4 (IgG3) (Developmental Studies Hybridoma Bank, Iowa City) were detected with a FITC-labeled goat anti-mouse IgG and IgM antibody (Caltag, Burlingame, CA). Other conjugated antibodies were used: CD34-FITC, CD45-PE, CD31-PE, CD38-PE, CD90-FITC, CD117-PE, CD15-FITC, class I-FITC (all IgG1, all from PharMingen), CD133/1-PE (IgG1) (Milenyi Biotec, Auburn, CA), and glycophorin A-PE (IgG1) (Immunotech, Miami, FL). Cells were analyzed live (without fixation) by using propidium iodide to exclude dead cells on a FACScan (Becton Dickinson) with either PC-LYSIS or CELLQUEST software.

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**Magnetic Column (MACS) Separation.** Selection of CD34<sup>+</sup> cells was done by labeling the H1/S17 cells with the anti-CD34 antibody QBEND/10 followed by a magnetically labeled secondary antibody (Miltenyi Biotec). The magnetically labeled cells were separated into CD34<sup>+</sup> and CD34<sup>-</sup> populations with a mini-MACS column (Miltenyi Biotec). CD34 enrichment was confirmed by flow cytometry analysis using a different anti-CD34 antibody (PharMingen).

**Hematopoietic Colony Assays.** H1/S17 cells, H1/C166 cells, or H1/MEF cells were cultured for the indicated number of days before harvesting and making a single cell suspension as above. Hematopoietic colonies were demonstrated by growing these cells in Methocult GF+ media (StemCell Technologies, Vancouver) consisting of 1% methylcellulose, 30% FBS, 1% BSA, 50 ng/ml stem cell factor, 20 ng/ml granulocyte-macrophage colony-stimulating factor, 20 ng/ml IL-3, 20 ng/ml IL-6, 20 ng/ml granulocyte colony-stimulating factor, and 3 units/ml erythropoietin. Cells were aliquoted in duplicate samples at  $1-2 \times 10^5$  cell per plate. After 14 days the plates were scored for colony-forming units (CFUs) according to standard criteria (20, 21). To demonstrate CFU-megakaryocyte (CFU-Mk) colonies, the H1/S17 cells were cultured on chamber slides in MegaCult-C media (StemCell Technologies) consisting of 1.1% collagen, 1% BSA, 10  $\mu$ g/ml bovine pancreatic insulin, 200  $\mu$ g/ml human transferrin, 2 mM L-glutamine, 0.1 M  $\beta$ -mercaptoethanol, 50 ng/ml thrombopoietin, 10 ng/ml IL-6, and 10 ng/ml IL-3. The Megacult-C media were supplemented with 40  $\mu$ g/ml low density lipoproteins (Sigma) as recommended by the manufacturer. After 10-14 days, the cells were fixed, dried, and stained with an anti-CD41 (GPIIb)-specific antibody or isotype control antibody, followed by an alkaline phosphatase-conjugated secondary antibody and visualization with Fast Red/Naphthol staining according to the manufacturer's instructions. CFU-Mk cells were identified by red staining.

Cellular morphology and enzyme expression were examined by plucking individual colonies with a pulled Pasteur pipette and spinning onto glass slides by using a Cytospin 2 (Shandon, Pittsburgh). Cells either were stained with Diff-Quik (a modified Wright-Giemsa stain, Dade Behring, Miami) or stained for esterase-containing cells with Naphthol AS-D Chloroacetate esterase and  $\alpha$ -Naphthyl acetate esterase (Sigma).

**Reverse Transcriptase-PCR (RT-PCR) Analysis.** Cells used for initial RT-PCR studies were: H1 cells allowed to differentiate on S17 cells (H1/S17), H1 cells allowed to differentiate on MEFs (H1/MEF day 17), H1 cells on MEFs for 6 days and harvested before differentiation was seen (H1/MEF day 6), irradiated S17 cells alone, irradiated MEF cells alone, and the erythroleukemia cell line K562 (American Type Culture Collection). Adherent cells were harvested with 1 mg/ml collagenase IV, washed with PBS, and pelleted. Total RNA was extracted by using a RNeasy mini kit (Qiagen, Valencia, CA) with homogenization with a Qiasredder (Qiagen) according to the manufacturer's instructions. Total RNA was quantified by UV spectrophotometer and 1  $\mu$ g was used for each RT reaction. For time-course experiments, 0.5  $\mu$ g RNA was used for each RT sample. RT reactions were done by using Omniscript RT (Qiagen) according to the manufacturer's instructions. Duplicate samples with and without addition of RT enzyme were done for all studies to control for contaminating genomic DNA. RT reactions were primed by using oligo(dT) primers (Promega), and 20 units RNase inhibitor was added to each reaction (Promega). PCRs were done with HotStarTaq (Qiagen) using 2  $\mu$ l of RT product per reaction according to the manufacturer's instructions. PCR conditions consisted of: 15 min at 95°C (hot start), 25-40 cycles

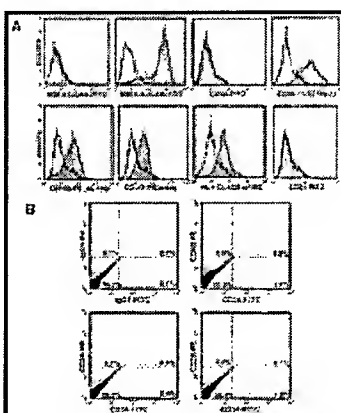


(actual number noted below) of: 94°C for 1 min, annealing temperature ( $T_a$ , noted below) for 1 min, 72°C for 1 min. A final 10-min extension at 72°C was done at the end. Oligonucleotide-specific conditions were: TAL-1, 40 cycles,  $T_a$  53°C; GATA-2: 31 cycles,  $T_a$  53°C; Flk-1, 35 cycles,  $T_a$  53°C; LMO-2, 40 cycles,  $T_a$  53°C; and  $\beta$ -actin, 25 cycles,  $T_a$  58°C. Products were analyzed on 1.5% agarose gel and visualized with ethidium bromide staining. DNA sequencing was done to confirm bands corresponded with the appropriate human genes. Oligonucleotide primers were: TAL-1 (331 bp), forward, 5'-ATGGTGCAGCTGAGTCCTCC-3', reverse, 5'-TCTCATCTTGCTGAGCTTC-3'; GATA-2 (242 bp), forward, 5'-AGCCGGCACCTGTTGTGCAA-3', reverse, 5'-TGACTTCTCCTGCATGCACT-3'; Flk-1 (537 bp), forward, 5'-ATGCACGGCATCTGGGAATC-3', reverse, 5'-GCTACTGTCCTGCAAGTTGCTGTC-3'; LMO-2 (289 bp), forward, 5'-GGATCCTGCCGGAGAGACTATCTC-3', reverse, 5'-GAATTCAGTGAACACCTCCGCAAA-3'; and  $\beta$ -actin, (838 bp), forward, 5'-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3', reverse, 5'-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3'.

## ► Results

**Hematopoietic Differentiation of Human ES Cells.** The majority of these experiments were done by using the human ES cell line H1 (14). These cells were maintained in the undifferentiated state by coculture on irradiated MEF "feeder cells" in serum-free media. Flow cytometric analysis of undifferentiated H1 cells demonstrates they are SSEA-1<sup>-</sup>, SSEA-4<sup>+</sup>, as previously demonstrated by immunohistochemical staining (14) (Fig. 1*A*). Here, we further characterized these human undifferentiated ES cells as expressing CD90 (thy-1), CD133 (AC133), and CD117 (*c-kit*). However, H1 cells fail to express CD34, CD31, CD45, and CD38 (Fig. 1*A* and data not shown). Interestingly, CD90, CD133, and CD117 (*c-kit*) are well recognized to be present on HSCs, and recently CD133 was identified on purified human neural stem cells (7, 22, 23).

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**Fig. 1.** Flow cytometric analysis of undifferentiated human ES (H1) cells and differentiated H1 cells. (*A*) Undifferentiated H1 cells analyzed by single-color flow cytometry. Appropriate isotype control antibody is demonstrated by line and indicated antibody by filled plot. SSEA-1 and SSEA-4 were unconjugated antibodies and a secondary FITC-conjugated goat anti-mouse (GAM) antibody was used. All other antibodies were directly conjugated to fluoro-chrome. (*B*) H1 cells allowed to differentiate on S17 cells (H1/S17 cells) analyzed by two-color flow cytometry. Percentages of positive cells are indicated in each quadrant.

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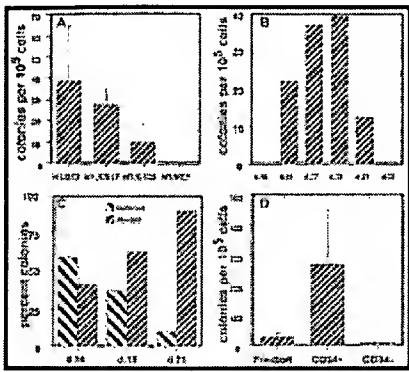
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To promote hematopoiesis, the undifferentiated H1 cells were cocultured with irradiated S17 cells (originally derived from mouse bone marrow; ref. 18), or with C166 cells (originally derived from embryonic day 12 mouse yolk sac; ref. 19). The media contained 20% FBS, but no other exogenously added cytokines or growth factors. Both the S17 and C166 cell lines have been shown to support the growth of bone marrow-derived hematopoietic progenitor cells (24, 25). After 3-5 days in culture under these conditions, the H1 cells differentiated into a variety of cell types. Within these areas of differentiation were regions of cobblestone-type cells and other areas of small, round loosely adherent cells. The appearance of these cells is reminiscent of early hematopoietic cells derived from other sources (7).

Initially, to characterize potential hematopoietic cells, the H1 cells allowed to differentiate on either S17 cells (H1/S17 cells) or C166 cells (H1/C166 cells) for 17 days were analyzed by flow cytometry (Fig. 1B). Approximately 1-2% of the differentiated H1 cells were shown to be  $CD34^+CD38^-$ , consistent with the phenotype of early hematopoietic cells (7, 26). Interestingly, roughly 50% of the  $CD34^+$  cells also expressed CD31 on the cell surface. Other studies have shown that CD34 and CD31 can be coexpressed on both HSCs and endothelial cells, and both cell types are thought to be derived from the same hemangioblast precursor cells (27-29). Therefore, it is possible that endothelial cells or endothelial precursors are also present within these cultures of differentiated H1 cells. These  $CD34^+CD38^-$  cells also were found to be  $CD45^-$  (Fig. 1B). Although CD45 is commonly expressed on mature hematopoietic cells, expression of CD45 on HSCs and hematopoietic colony-forming cells (CFCs) is unclear. Studies of both human and murine hematopoiesis have identified  $CD45^-$  hematopoietic precursors. This includes work on differentiated mouse ES cells and day 9.5 mouse embryonic yolk sac that demonstrate hematopoietic CFCs from  $CD45^-$  cell populations (5).

**Hematopoietic Colony Assays.** Cells that form hematopoietic colonies (so-called CFUs or CFCs) represent a stage of hematopoietic differentiation between HSCs and more terminally differentiated cells (such as erythrocytes, granulocytes, monocytes, or platelets). These CFCs are identified by culturing them in a semisolid media (typically methylcellulose or agar) supplemented with cytokines that promote the localized expansion and differentiation of hematopoietic cells in discrete colonies. In methylcellulose assays, on average, H1/S17 cells gave rise to 30.4 colonies per  $10^5$  input cells, and H1/C166 gave rise to 4.3 colonies per  $10^5$  input cells (Fig. 2A). H1/S17 cells produced CFCs after 14 days of coculture (but not at earlier times), produced a maximal number of CFCs at 17-18 days, and produced no CFCs at 28 days (Fig. 2B). This finding demonstrates the transient nature of hematopoietic differentiation within this system and suggests that long-term self-renewal of HSCs is not supported by this stromal coculture method. The clonally derived human ES cell lines H1.1 and H9.2 gave similar results when allowed to differentiate on S17 cells (Fig. 2A and data not shown). Importantly, none of the following conditions lead to generation of CFCs: undifferentiated H1 cells (harvested after 6 days on MEFs without evidence of differentiation), H1 cells allowed to differentiate on MEF cells for 17 days (H1/MEF), H1 cells allowed to differentiate on S17 cells in serum-free media, and S17 cells or C166 stromal cells alone (Fig. 2A and data not shown). H1/S17 cells and H1/C166 cells gave rise to both erythroid and myeloid (nonerythroid) colonies (Fig. 2C). Interestingly, after 14 days of differentiation H1/S17 cells produced mostly erythroid CFCs, whereas after 21 days of differentiation H1/S17 cells produced mostly myeloid CFCs (Fig. 2C).

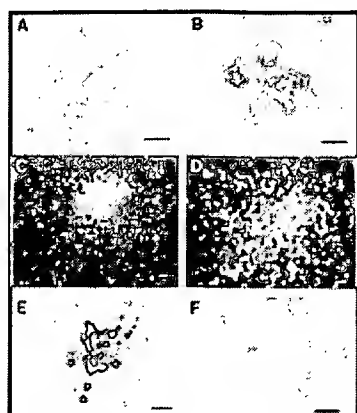


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**Fig. 2.** Methylcellulose hematopoietic colony-forming assays. (A) Production of CFCs from human ES cells. H1/S17 cells, H1.1/S17, H1/C166 cells, and differentiated H1/MEF cells were harvested after 14–20 days of culture, placed in methylcellulose-based media supplemented with hematopoietic growth factors, and scored for total hematopoietic colonies after 14 days. Results are mean  $\pm$  SE of seven trials with H1/S17, four trials with H1.1/S17, four trials with H1/C166, and three trials with H1/MEF. Data are presented as colonies per  $10^5$  cells harvested from the differentiated H1 cultures. (B) Time course of H1/S17 cell differentiation into hematopoietic CFCs. H1 cells cocultured with S17 cells for the indicated number of days before colony assay. (C) Percent of erythroid (burst-forming unit-erythroid) and myeloid (CFU-GM, CFU-M, and CFU-G) colonies derived from H1/S17 cells harvested at day indicated. (D) CD34<sup>+</sup> H1/S17 cells are enriched for CFCs. Unsorted H1/S17 cells and H1/S17 cells sorted for CD34<sup>+</sup> cells and CD34<sup>-</sup> cells by magnetic column were placed in hematopoietic colony assay. These results are mean  $\pm$  SE of three separate trials.

Because CD34 is the best identified surface antigen expressed on hematopoietic precursor cells, the H1/S17 cells were enriched for CD34<sup>+</sup> cells by magnetic selection. Here, the CD34-enriched cells gave rise to on average 270 colonies per  $10^5$  H1/S17 cells and the CD34-depleted cells gave rise to only 10 colonies per  $10^5$  H1/S17 cells (Fig. 2D). Therefore CD34 selection markedly enriched CFCs compared with the unselected H1/S17 cells, whereas the CD34-depleted cell population was reduced in CFCs. Because the CD34-depleted cells still contained  $\approx 0.5\%$  CD34<sup>+</sup> cells (postdepletion), the CFCs from the CD34-depleted cells may have come from either CD34<sup>-</sup> cells or contaminating CD34<sup>+</sup> cells.

The colonies formed in methylcellulose had the same highly characteristic morphologies of colonies derived from human bone marrow cells placed in similar culture conditions (Fig. 3A–E)(20, 21). The phenotypes of the CFCs included CFU-macrophage (CFU-M), CFU-granulocyte, mixed CFU-macrophage/granulocyte (CFU-GM), burst-forming unit-erythroid, and CFU-erythroid. Occasional experiments also demonstrated mixed CFU-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM), an early multipotent progenitor cell. CFU-Mks were demonstrated by culture of the H1/S17 cells in a collagen based-media designed to support CFU-Mk growth. The CFU-Mk were specifically identified by immunostaining with an antibody against CD41 (GPIIb of the GPIIb/IIIa complex), specific for megakaryocytes and platelets (Fig. 3E). Staining of the CFU-M- and CFU-GM-derived colonies with nonspecific esterase demonstrated granules typical of these lineages (Fig. 3F). Mature neutrophils also could be identified within the CFU-GM-derived cells by their typical nuclear morphology (Fig. 3F). Flow cytometric analysis of cells within these colonies demonstrates expression of surface antigens typical of normal human blood cells. These cells are CD45<sup>+</sup>, HLA class I<sup>+</sup>, and CD34<sup>-</sup>. The erythroid cells express glycophorin A, and the myeloid cells express CD15 (Fig. 4).

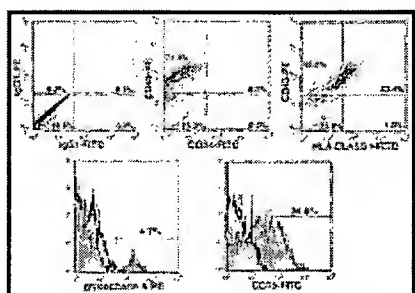


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**Fig. 3.** Photographs of hematopoietic colonies and cells derived from H1/S17 cells. H1 cells allowed to differentiate on S17 cells for  $\approx 17$  days, harvested, and allowed to form colonies in semisolid media for 14 days before scoring colony phenotypes. (A) CFU-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM). Colony of mixed erythroid and myeloid cells. (B) Burst-forming unit-erythroid. Large unstained, red (hemoglobin) colony. (C) CFU-GM, unstained myeloid colony. (D) CFU-M, unstained myeloid colony, less dense than CFU-GM colony. (E) CFU-Mk. Colony of cells stained with platelet/megakaryocyte-specific antibody against CD41 (GPIIb/IIIa) with alkaline phosphatase-conjugated secondary antibody and Fast Red/naphthol reagent to provide red stain. (F) Cytospin of CFU-GM cells demonstrating granulocytes with esterase-positive red granules. (Scale bars: A-D, 100  $\mu$ m; E, 40  $\mu$ m; F, 20  $\mu$ m.)



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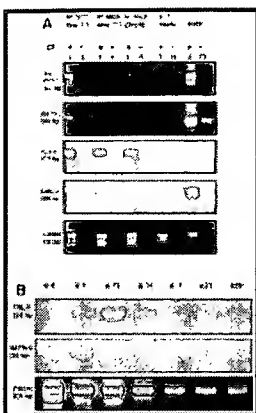
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**Fig. 4.** Flow cytometric analysis of hematopoietic cells derived from methylcellulose colony assay of H1/S17 cells. Cells were washed free of methylcellulose before incubation with indicated antibodies and analyzed by two-color (*Upper*) or one-color (*Lower*) flow cytometry. (*Upper*) Isotype controls are shown (*Left*), and percent positive cells in each quadrant is indicated. (*Lower*) Isotype control is demonstrated by line, and indicated antibody is demonstrated by filled plot. Percent positive cells are shown by labeled marker.

**Hematopoietic Gene Expression.** To further characterize the H1 cells differentiated to hematopoietic cells by coculture with S17 cells (H1/S17 cells), we examined genes known to be expressed at an early stage of hematopoietic differentiation by using RT-PCR. H1/S17 cells expressed mRNA for TAL-1 and GATA-2, confirming the presence of early hematopoietic cells uniquely within this population (Fig. 5A). The vascular endothelial growth factor receptor Flk-1 and the transcription factor LMO-2 were expressed in undifferentiated H1 cells, H1/S17 cells, and H1/MEF cells. This finding suggests that Flk-1 and LMO-2 may have important roles in cells other than hematopoietic cells, including undifferentiated ES cells. The S17 and MEF feeder cells alone do not express any of these genes (Fig. 5 and data not shown). Time-course analysis found expression of TAL-1 and GATA-2 as early as day 7, before the appearance of CFCs. However, expression of GATA-2 was not detectable after day 21, corresponding to loss of CFC generation (Fig. 5B).

**Fig. 5.** Hematopoietic gene expression by RT-PCR of H1/S17 cells. (A)



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H1 cells were allowed to differentiate on either S17 cells for 17 days (lanes 1 and 2) or to differentiate on MEF cells for 17 days (lanes 3 and 4), or harvested after culture on MEFs for 6 days, before evidence of differentiation (undifferentiated H1 cells, lanes 5 and 6) and subjected to RT-PCR analysis. Irradiated S17 cells were examined to demonstrate positive bands in the H1/S17 samples were not from these feeder cells (lanes 7 and 8). The erythroleukemia cell line K562 was used as a positive control (lanes 9 and 10). Oligonucleotide primers specific for genes of interest are shown. Each sample was done with RT added (+, lanes 1, 3, 5, 7, and 9) and without RT added (–, lanes 2, 4, 6, 8, and 10) to demonstrate positive bands are not caused by genomic DNA. (B) Time course of hematopoietic gene expression. H1 cells were allowed to differentiate on S17 cells for the number of days indicated prior isolation of RNA for RT-PCR analysis. Day 0 (d. 0) indicates undifferentiated H1 cells. Controls of PCR done on samples without RT added did not have any positive bands (data not shown).

Because the ES cell-derived erythroid colonies could potentially express globin genes from any stage of development (embryonic, fetal, or adult), we used RT-PCR to evaluate this gene expression. RNA was prepared from erythroid colonies harvested from methylcellulose culture of differentiated H1/S17 cells and compared with erythroid colonies formed by normal adult bone marrow. As expected, the adult bone marrow-derived colonies expressed ample  $\alpha$  and  $\beta$  RNA, as well as some  $\gamma$  and  $\delta$  RNA. In contrast, the H1/S17-derived colonies also expressed  $\alpha$ ,  $\beta$ , and  $\delta$  globin, but did not express fetal  $\gamma$  globin. No embryonic ( $\epsilon$  or  $\zeta$ ) globin gene expression was detected (data not shown). These results show that the ES cell-derived erythroid cells can express mature, adult-type hemoglobin.

## ► Discussion

We have demonstrated *in vitro* differentiation of human ES cells to multiple hematopoietic lineages. Although *in vitro* colony assays are commonly used to study human hematopoiesis (30, 31), one concern about using colony assays to identify ES cell-derived hematopoietic cells is that the colonies could consist of nonhematopoietic cells that are able to grow in clusters that merely resemble hematopoietic colonies.

However, we have used several complementary methods to demonstrate that these colonies consist of hematopoietic cells. The hemoglobin (red) of the burst-forming unit-erythroid colonies provide a distinct marker of terminally differentiating erythroid cells. Moreover, these cells are glycophorin A<sup>+</sup> and express normal adult globin genes as detected by RT-PCR. The esterase-positive granules in the CFU-GM-derived colonies are characteristic of granulocytes and macrophages. Additionally, the cells within the myeloid-derived (CFU-GM and CFU-M) colonies are CD45<sup>+</sup> and CD15<sup>+</sup>. The CFU-Mk-derived cells (megakaryocytes) are CD41<sup>+</sup>. Sorting the H1/S17 cells into CD34<sup>+</sup> and CD34<sup>–</sup> populations demonstrates enrichment of CFCs within the CD34<sup>+</sup> population, as expected for hematopoietic precursors. Although the frequency of CFCs is relatively low under the conditions of differentiation described, the yield using CD34-enriched populations is close to the number of CFCs

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derived from human bone marrow samples ( $\approx 100$ -1,000 CFCs per  $10^5$  bone marrow cell). Further studies to evaluate methods to derive more highly purified populations of HSCs and CFCs from human ES cells will be of interest.

The hematopoietic differentiation of human ES cells has important therapeutic implications, including the derivation of erythrocytes and platelets for transfusions, and the derivation of HSCs for hematopoietic cell transplantation. Because ES cells can be expanded without apparent limit (16), ES cell-derived blood products could be created in virtually unlimited amounts. These cells could be screened for pathogenic organisms and even potentially be genetically engineered to treat specific patients or to combat specific diseases. ES cell-derived HSCs could dramatically increase both the availability and the effectiveness of HSC transplantation for the treatment of hematologic malignancies. Recent work in mice suggests that highly purified HSCs can provide long-term engraftment across complete allogeneic barriers (32, 33). However, the dose of purified HSCs required to obtain engraftment across these allogeneic MHC barriers is high. By using human ES cells as the starting cell population, a sufficiently large dose of pure HSCs could be generated, which would permit allogeneic engraftment. Importantly, the *in vitro* derivation of HSCs capable of long-term, multilineage engraftment from mouse ES cells has so far proven an elusive goal. Mouse ES cells, however, clearly have this potential, as they routinely contribute to the definitive hematopoietic system *in vivo* when formed into chimeras with preimplantation embryos (34). Thus, the failure of hematopoietic cells derived *in vitro* from mouse ES cells to support long-term, multilineage engraftment reflects our current ignorance of hematopoietic differentiation, but does not reflect a defect in the developmental potential of ES cells.

The derivation of engraftable HSCs from human ES cells will have implications for human medicine far beyond the treatment of hematologic malignancies, as these HSCs may provide a powerful method to prevent immune rejection of other ES cell-derived tissues (35). Use of hematopoietic cell transplantation as a means to create tolerance to a solid organ transplant has been studied since the 1950s (36). Recent studies have shown that transplantation of highly purified mouse HSCs across allogeneic barriers creates tolerance to other tissues that share the same genetic background as the HSCs (37). Other studies in both primates and humans demonstrate that hematopoietic chimerism can create a state of tolerance that permits long-term survival of transplanted organs without continued immunosuppression (38, 39). If human ES cell-derived HSCs can be used to create hematopoietic chimerism in a patient, that patient should be tolerant to other tissues derived from the same ES cells and would not require any continuous immunosuppressive treatment.

The clinical promise of human ES cell-based therapies is great; however, because these therapies will be entirely novel, serious concerns about safety and efficacy will need to be addressed before human clinical trials can be initiated. The malignant transformation of cells that have been cultured for extended periods is a particular concern. Because we also have isolated ES cells from rhesus monkeys (40), it will be possible to use these primate cells as an accurate, preclinical transplantation model for human ES cell-based therapies. Recently, the hematopoietic potential of the rhesus monkey ES cells has been demonstrated (41). These animal models, as well as continued study of methods to promote lineage specific differentiation, will facilitate the potential clinical applications of human ES cell-based therapies.

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## ► Abbreviations

ES, embryonic stem; HSC, hematopoietic stem cell; MEF, mouse embryonic fibroblast cell; CFC, colony-forming cell; RT-PCR, reverse transcriptase-PCR; PE, phycoerythrin; CFU, colony-forming unit; CFU-Mk, CFU-megakaryocyte; CFU-M, CFU-macrophage; CFU-G, CFU-granulocyte; CFU-GM, CFU-macrophage/granulocyte.

## ► Footnotes

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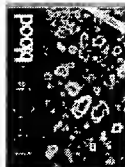
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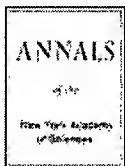
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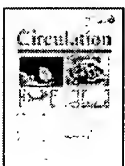
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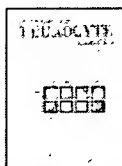
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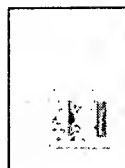
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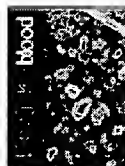
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